Genome variations in transgenic tobacco explored by representational difference analysis

Chapter 1: Plant genome variation under stress

Any change in an environmental condition reducing or adversely modifying growth and development of an organism can be considered as stressful, and potentially affecting the genome of an organism. In comparison to the great number of reports focusing on variable gene expression under stress, investigations about genomic variation at the DNA sequence level as a response to stress are rather limited. This introductory review outlines the current knowledge about stress-induced genome variations mainly investigated in plant tissue culture and their detection on the DNA sequence level. Variation includes both direct changes in the DNA sequence and epigenetic variation due to DNA methylation. Detection of such genome variations and possible related phenotypic changes have been investigated with a variety of test methods at the morphological, cytological, cytochemical, biochemical, and molecular levels.

I) The plant genome and stressful environments

Conditions for growth are seldom optimal and any change in an environmental condition that results in a response of an organism might be considered as stressful with the potential for modifying genome composition, growth and development of the organism (Levitt, 1972; Koehn and Bayne, 1989).

Many research groups have investigated the stressful factors that vary gene expression. Typical environmental stress factors *in vivo* include pathogenic micro-organisms, a wide variety of abiotic stresses, such as drought, extreme temperatures, toxic minerals and pollutants, and also *in vitro* plant processes such as tissue culture and genetic engineering of plants (Chapin, 1991;

Fowden et al., 1993; Ditt et al., 2001; Cassells and Curry, 2001). Figure 1.1 outlines a variety of stresses affecting the plant genome.

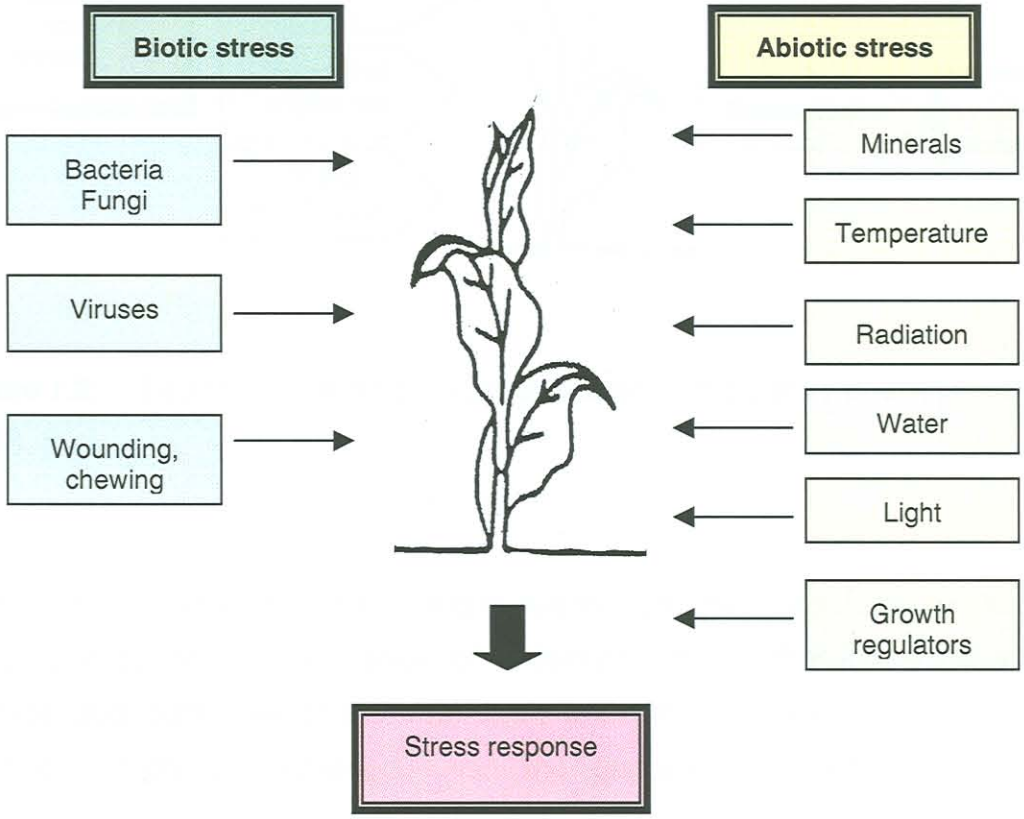

Figure 1.1: Abiotic and biotic stresses affecting the plant genome (Salisbury and Ross, 1992).

II) Stress and the response of the plant genome

A plant needs to adapt to stress in order to survive and many factors determine how the plant genome responds to stress. The genetic make-up of a plant, its developmental circumstances, the duration and severity of the stress, the number of times the plant is subjected to stress and any synergistic effects of multiple stress influences this genome response (Figure 1.2). If adaptation and repair mechanisms are not sufficient and the effect of the stress factors is ultimately not alleviated, the outcome of stress will be death of the organism.

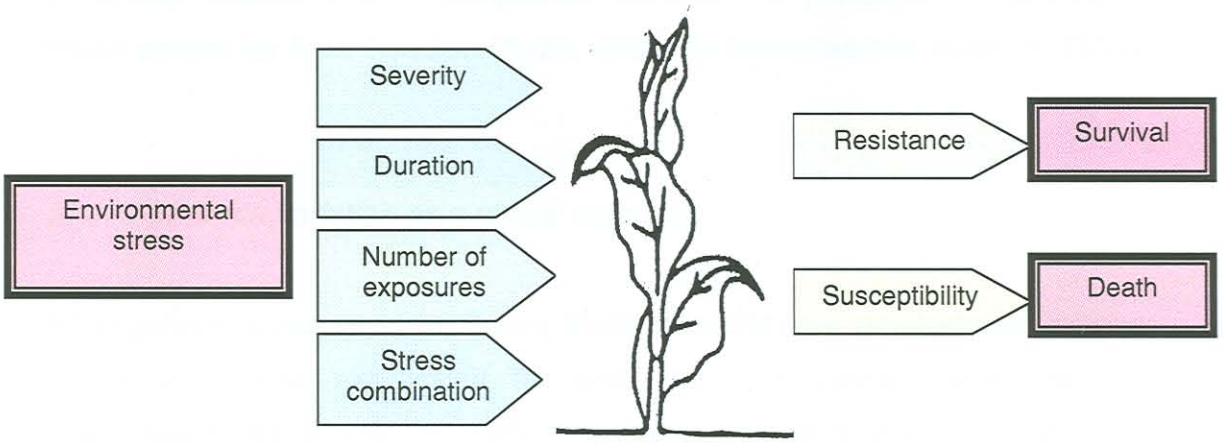

Figure 1.2: Factors determining the stress response of a plant (Buchanan et al., 2000).

Plants can survive by either avoiding or tolerating stress. Simple avoidance of stress can be through expression of certain phenotypic characteristics, such as extended roots down to the water table to avoid drought stress. Tolerance of stress might be achieved when the genome expresses temporarily protective proteins to modulate metabolism such as the heat shock proteins. A large number of research groups have investigated variable gene expression of protective systems under stress, including the function of a single protective gene in transgenic plants, or production of a complete set of new proteins, such as heat shock proteins (Viswanathan and Khannachopra, 1996; Sabehat et al., 1998; Smirnoff, 1998; Bartels and Nelson, 1994; Savenstrand et al., 2002; Savenstrand et al., 2000). Among the specific targets for investigation of up-regulation of protective systems by the genome are pathogenesis-related proteins and components of the cellular antioxidative system, which protect against oxidative stress (Kitajima and Sato, 1999; Foyer and Noctor, 2000; Foyer et al., 1994; Perl-Treves and Perl, 2002). Protective systems are sometimes transmitted to and expressed in distant tissues and even in neighboring plants. For example, the chewing of insects or wounding activates proteinase inhibitors that initially release a signal molecule, such as systemin, leading to a signaling cascade that

eventually results in a transcriptional activation of proteinase inhibitors for insect control far from invasion (Ryan, 2000; de Bruxelles and Roberts, 2001).

A) Genome variation as a stress response

An organism is prepared for facing many stresses in a programmed manner by variable gene expression for protection. However, there are also responses of the genome to challenges that are not so precisely programmed. This might result in chromosome breakage, DNA mutations and ultimately changed gene expression (McClintock, 1984). In comparison to the great number of reports focusing on variable gene expression under stress, fewer reports about the direct effects of stress on the DNA sequence are available. Several mechanisms, such as quantitative modification of repetitive DNA, DNA methylation, excision and insertion of transposable elements, gene amplification or deletion and histone acetylation have been suggested as points of control on the DNA sequence level for these challenges (Figure 1.3; Capy, 1998; Cullis, 1990; Johnston et al., 1996).

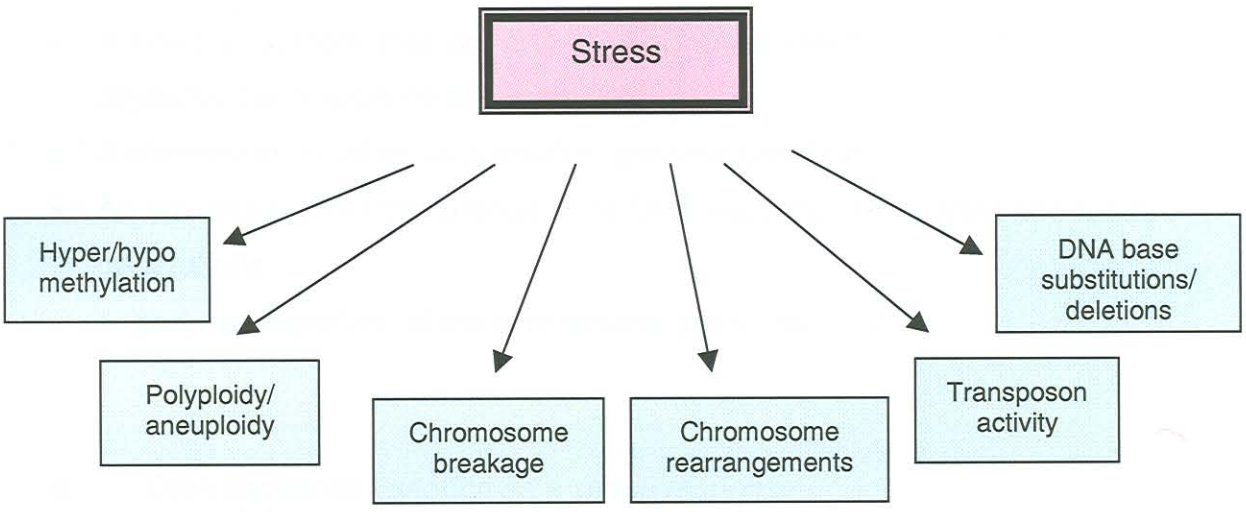

Figure 1.3: Stress and genome modifications (Cassels and Curry, 2001).

The genome has been considered for long as stable to stress despite occasional changes in chromosome structure or inversions. However, recent research suggests that the genome is rather flexible and can undergo changes, which are often referred to as plasticity (Capy, 1998). Such changes might occur naturally over long time periods during evolution. But plant tissue culture, where plants are rapidly propagated in a stressful, artificial environment, seemingly induces such changes in a relatively short time period. Chromosome rearrangements were detected in regenerated tissue culture plants of *Zea mays* (Lee and Phillips, 1986) and changes in the amount of rDNA and peroxidase isozyme band patterns were found in flax exposed to stress (Cullis, 1981). Since plants are unable to move and search for favourable growth conditions, they have to adapt their genome to the changing environment. Walbot and Cullis (1983 and 1985) proposed that once the ordinary physiological responses to an environmental stress are exhausted, the plant genome has to adapt to the new environment by rearranging its DNA in limited genomic regions, which might be related to phenotypic effects. According to Cullis (personal communication), the genomic response in plants needs to have the following properties to have an adaptive advantage towards stress:

- A sensing system that activates the adaptive mechanism when normal physiological responses are exhausted.
- A phenotypic variation as a result of genome variation.
- An advantage from the change in its DNA sequence for current and future generations.
- A genome alteration, which is reversible and limited.

B) DNA sequence variation as a stress response

Genome variation in plants as a response to stress can be either genetic or epigenetic. Figure 1.4 summarizes the genetic and epigenetic genome variations, which have been reported (Cassells and Curry, 2001; Kaeppler et al., 2000; Abe et al., 2002). Genetic changes include both chromosomal gross

rearrangements and changes in the DNA sequence, whereas epigenetic changes are primarily alterations in DNA methylation. All parts of the genome may not be equally susceptible so that variation in the genome is dependent on a particular stress being experienced. However, some regions in the DNA sequence of the genome may be more susceptible during stress than others and therefore might alter irrespective of the inducing stress. Plant tissue culture has been investigated as a stressful process in greater detail for these variable regions, due to its commercial implications.

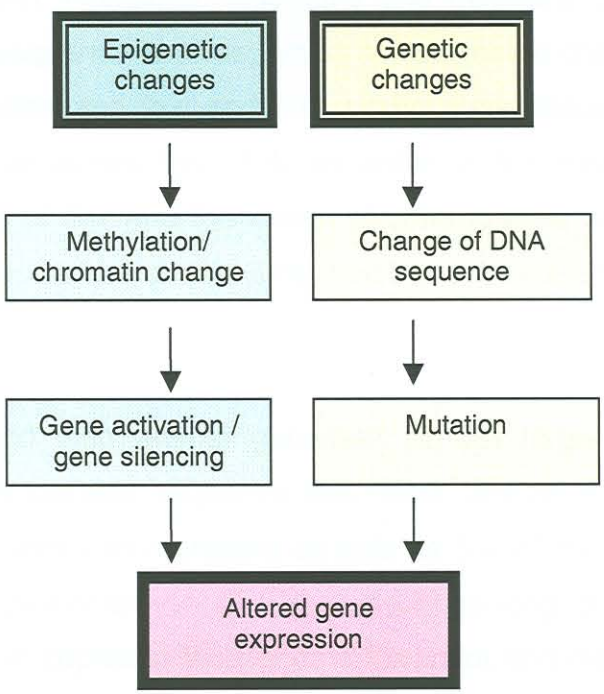

Figure 1.4: Genetic and epigenetic changes in plants due to stress (Capy et al., 2000; Nakao, 2001)).

Genomic variation in tissue culture can result in aneuploidy, chromosomal rearrangements, activation of transposable elements, point mutations, genome rearrangements, changes in ploidy level, methylation changes and even altered copy number of sequences (Cullis, 1990; Peschke et al., 1987; Hirochika, 1993; Phillips et al., 1994). Changes in the ploidy level and

chromosome rearrangements are quite common in tissue culture plants (Kaeppler et al., 2000). In barley ploidy changes were the most prevalent cytological changes followed by chromosome breakage (Hang and Bregitzer, 1993). There is evidence that late replication of hetero-chromatin in tissue culture plants might cause chromosome breakage (Johnson et al., 1987). McCoy et al. (1982) performed a meiotic analysis on regenerated oat plants and found loss of part or the entire chromosome arms, and chromosomal instabilities were also found among *in vitro* grown maize plants (Lee and Phillips, 1986). Translocations were a commonly observed chromosome abnormality with inversions, insertions and deletions occurring in the DNA sequences. Sequence families, which are subject to change can include both repetitive families and low and / or single copy sequences. Blundy et al. (1987) found an almost three-fold reduction in the ribosomal RNA genes in callus cultures of flax and the extent of chromosome abnormalities in maize culture was dependent on the time isolated cells were cultured (Chandler et al., 1986).

Most plant and also animal genomes consist largely of repetitive DNA. Stretches of nucleotide sequence that occur one or only a few times in the genome of a plant can represent as little as 5% of the DNA, while repetitive sequences, typically one to 10 000 nucleotides long, are present in hundred or thousands of copies in the genome (Schmidt and Heslop-Harrison, 1998). Repetitive DNA sequences are especially sensitive to stress-related DNA changes and account for a large portion of variation in sequence copy numbers. Plant tissue culture for example influences such repetitive DNA sequences. *Cymbidium* protocorms exposed to a chemical stress using an auxin-type plant growth regulator, amplified AT-rich satellite DNA, whereas exposure to the plant hormone gibberellic acid increased a GC-rich fraction (Nagl and Rucker, 1976). Highly repeated sequences were amplified up to 75-fold in rice suspension cultures (Zheng et al., 1987) and reduction in copy number of a highly repetitive DNA sequence in plant tissue culture of *Medicago* was also recently reported (Pluhar et al., 2001).

Ribosomal RNA sequences are another highly repetitive sequence family, which can be affected by stress (Blundy et al., 1987). Ribosomal RNA (rRNA) is transcribed from DNA as a large RNA precursor that is subsequently processed. Two types of ribosomes are known in higher plants termed the 70S and 80S (Ting, 1982). The 80S ribosomes are located in the cytoplasm and the 70S are located in the chloroplast and mitochondria. These ribosomes contain smaller subunits and are repeated and arranged in one or more tandem arrays (Nierras et al., 1997). With the exception of some legumes, almost all plant chloroplast genomes, including tobacco, contain two copies of a large inverted repeat, with a size of between 20 and 25 kb. The inverted repeat regions contain the 16S, 23S and 5S rRNA genes as well as some tRNA and ribosomal protein genes, and separate the large single-copy (LSC) and small single-copy regions (SSC) (Lu et al., 1996) (Figure 1.5). In contrast, the rRNA unit in the cytosol consists of the 18S, 5.8S and 25S rRNA coding regions with non-coding spacers with the 5S rRNA genes being present as tandem arrays elsewhere in the genome (Haberer and Fischer, 1996) (Figure 1.6). In the mitochondrion, rRNA is made up by the 18S, 5S and 26S coding units and non-coding spacers (Heldt, 1997). Copy numbers of rRNA genes are highly variable between plants species ranging from a few hundred to thousands of copies per haploid genome, for example *Linum usitatissimum* (flax) contains about a 1000 copies per haploid genome, while *Arabidopsis thaliana* contains about 570 repeats per haploid genome (Cullis, 1979; Pruitt and Meyerowitz, 1986). Some of these rRNA genes, such as the 5S rRNA gene, are highly conserved in the coding region and are useful tools to study evolutionary relationships in organisms. Although the ribosomal RNA sequences are highly variable, stress-induced DNA changes in these regions have not been investigated in great detail. So far, only a decrease in ribosomal RNA genes in callus culture of flax and changes in the amount of rDNA and peroxidase isozyme band patterns in flax exposed to stress have been reported (Cullis, 1981; Blundy et al., 1987). A study conducted by Bettini et al. (1998) also investigated tissue culture-induced variability of the rDNA in the presence or absence of stress but found no qualitative differences with either RFLPs or RAPDs.

Figure 1.5: Ribosomal RNA genes in the chloroplast: arrangement of the 16S-23S-5S RNA gene complex. Transcription of the 16S and 23S rRNA genes is from right to left. SSC = Small single copy region (Nierras et al., 1997; Haberer et al., 1996).

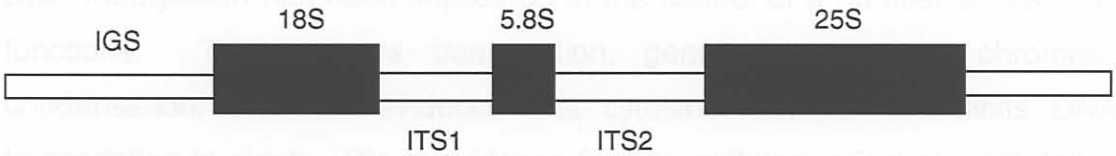

Figure 1.6: Ribosomal RNA genes in the cytosol: arrangement of the 18S-5,8S-25S RNA gene complex. IGS = intergenic spacer; ITS = internal transcribed spacer (Henry, 1997).

C) DNA methylation and stress

Epigenetic variation refers to any somatic or meiotic heritable alteration in gene expression, which is potentially reversible and not due to a DNA sequence change. Epigenetic variation involves mechanisms, such as gene silencing / activation, and can occur due to stress (Waddington, 1953; Kaeppler et al., 2000).

The most common epigenetic variation of DNA reported in plant cells is DNA methylation. Methylation occurs at carbon 5 of cytosines and up to 30% of the cytosine in plant DNA can be modified (Ohki et al., 2001). However,

levels of cytosine methylation can vary considerable between plant species. Rye (*Secale cereale*) has 33% methylation (Thomas and Sherratt, 1956) whereas *Arabidopsis thaliana,* with a small genome, has only 4.6% methylation (Leutwiler et al., 1984). In plants, 70-80% of the CG di-nucleotides are methylated and additional methylation is found for the tri-nucleotide CNG, where N can be any base (Finnegan et al., 1993). The CG dinucleotide has symmetrical cytosine residues in the two DNA strands and, when modified, both cytosines are methylated (Cedar et al., 1979). This symmetry allows the pattern of methylation to be maintained through DNA replication and 5-mC might allow interference with normal protein-DNA interactions and influence of gene expression.

DNA methylation has been implicated in the control of a number of genomic functions. This includes transcription, gene silencing and chromatin condensation. There is evidence that cytosine methylation inhibits DNA transcription in plants. Direct evidence for this inhibitory effect of methylation on transcription in plants comes from experiments in which *in vitro* methylated DNA was introduced into protoplasts of either tobacco or petunia (Weber and Graessmann, 1989). These experiments indicated that to inhibit gene expression the level of methylation must exceed a certain threshold and/or cover specific sites. Introduction of *in vitro* methylated DNA into the protoplasts of tobacco suppressed the expression of the *gus* gene under the control of the 35S promoter sequence from cauliflower mosaic virus (Hershkovitz et al., 1989). These losses of transcription in methylated regions can be due to either the prevention of transcription initiation or by impending transcript elongation. It is thought that this phenomenon serves as a genome-defence mechanism that guards against the deleterious effects of multicopy transposable elements and aberrant gene duplications.

Many studies also investigated the relation between methylation and inactivation of genes (Jost and Saluz, 1993). By linking methylation with ribosomal RNA genes in tobacco, Fulnecek et al. (1998) found that the 5S rRNA gene is highly methylated, exceeding the average methylation density of the tobacco genome. Methylation might also be involved in the control of

inactivation of different 25S rRNA domains in *Brassica* species (Chen and Pikaard, 1997) and by investigating the 5S RNA genes in soybeans, Quemada et al. (1987) demonstrated a decrease in methylation in newly initiated callus and suspension cultures. However, methylation patterns are not always altered. rDNA was quantitatively unchanged in methylation in crown gall callus of flax despite a dramatic reduction in the number of these sequences (Blundy et al., 1987).

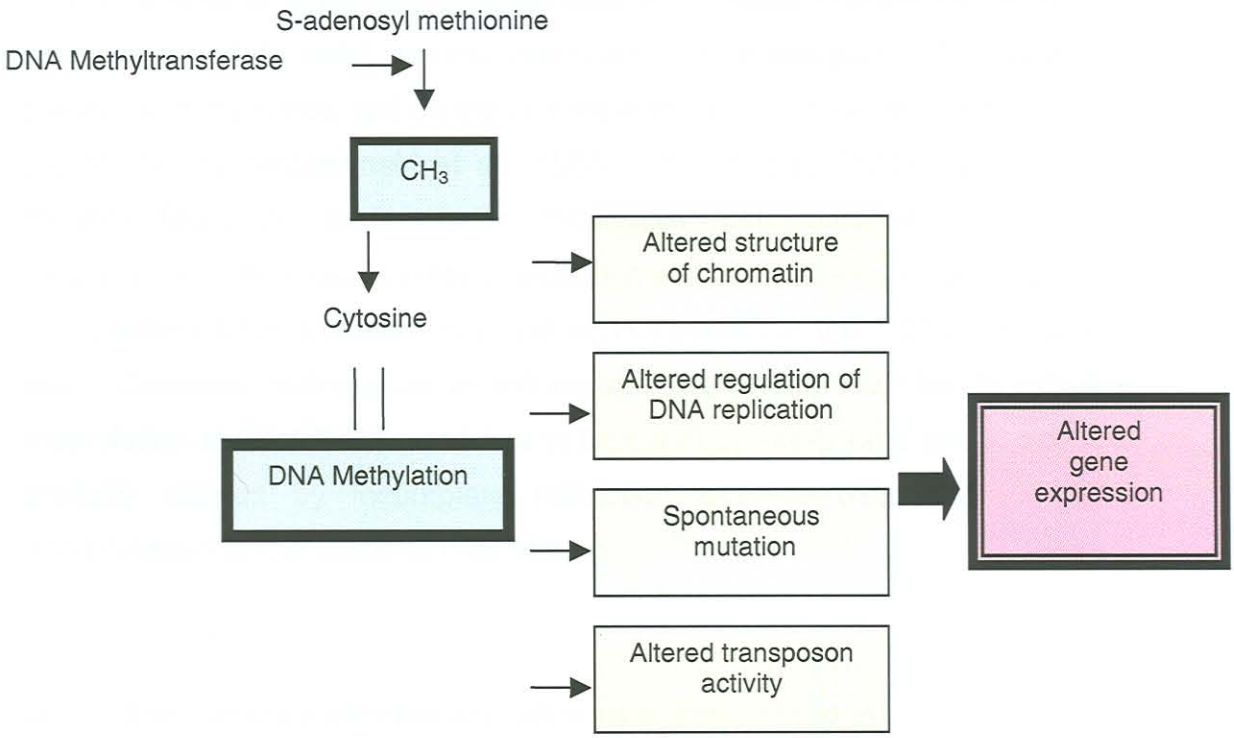

***Figure 1.7*:** DNA methylation in plants showing the enzyme DNA methyltransferase transferring a methyl group to cytosine residues and the resulting consequences for the plant (Nakao, 2001; Jost and Saluz, 1993).

Methylation is carried out by DNA methyltransferases. These enzymes catalyse the transfer of a methyl-group from S-adenosylmethionine to the pyramidine ring of cytosine residues (Figure 1.7). Plants have at least three classes of cytosine methyl-transferases, which differ in their protein structure and function (Nakao, 2001). The MET1 family most likely functions as

maintenance methyl-transferases, but they may also play a role in *de novo* methylation. The chromomethylases, which are unique to plants, seem to preferentially methylate DNA in heterochromatin. Putative *de novo* methyl-transferases finally represent the remaining class and the various classes of methyl-transferases show differential activity on cytosines (Nakano et al., 2000; Bender, 1998).

In plants, methylation is mainly restricted to the nuclear genome, where methylcytosine is especially concentrated in repeated sequences. However, controversial data exist on the methylation of chloroplast DNA in higher plants. A study conducted on the chloroplasts of peas detected methylation in the plastid (Ngernprasirtsiri et al., 1988). In contrast, Fojtova et al. (2001) recently found no evidence for chloroplast methylation in tobacco and *Arabidopsis*. The isoschizomeric restriction enzyme pair *Msp*I and *Hpa*II used for digesting DNA detected no possible methylation at the CCGG recognition site. Detected methylation in chloroplast DNA might be due to cytosine methylation at *Eco*RII sites and has to be carefully interpreted due to possible artifacts caused by incomplete restriction enzyme cleavage and false interpretation of low levels of methylation.

D) Transposable element activation as a stress response

Transposable elements can be activated in the plant genome by stress including plant tissue culture (McClintock, 1984; Peschke et al., 1987). Transposable elements are discrete sequences of DNA that are distinguished by their ability to move from one chromosomal site to another. Two families of transposable elements are known, the transposons and the retrotransposons. The retrotransposons, unlike the DNA transposons, such as the Ac transposon from maize (Brettell and Dennis, 1991), propagate not by "cutting and pasting", but by a mechanism of reverse transcription followed by integration of the new cDNA copy back into the genome (Boeke and Corces, 1989). Due to the replicative nature of retrotransposon mobilization combined with the large size of the elements, which is between 5 and 10 kb, these

elements have the potential to be major contributors to genome variation in stressful environments (Vicient et al., 2001). Larkin and Scowcroft (1981) proposed that the activation of transposable elements might be responsible for tissue culture-induced mutations and Hirochika et al. (1996) found that three of five reported rice retrotransposons were activated under tissue culture conditions and that their copy number increased with a prolonged culture period. Similar retrotransposon activation was also reported for tobacco (Hirochika, 1993) and transposition of the tobacco retrotransposon Tnt1 was observed in transformed *Arabidopsis* plants regenerated *in vitro* (Lucas et al., 1995).

There is further evidence that auxin-like compounds, such as 2,4-D, play a role in activating the promoter of the tobacco retrotransposon Tnt1 (Pauls et al., 1994) and that activity of transposons is related to DNA methylation, which is itself influenced by cellular stress (Banks et al., 1988). Brettell and Dennis (1991) reported that when plants containing a quiescent, unstable Ac element were cultured, the regenerated plants had a high frequency of element reactivation and Ac activation was related to the expected change in DNA methylation. Further, transposable elements are frequently present as dispersed repeats with up to 50 – 100 copies per cell (Sutton et al., 1984). Such a high copy number might also be one of the reasons why transposable elements are targeted for methylation.

Methylation of transposable element sequences can silence the expression of transposon-encoded genes and prevent transposon-mediated DNA re-arrangements. Due to cytosine methylation, a loss of RNA-polymerase-II-dependent transcription in the methylated regions is caused either by preventing transcription initiation or by impeding transcript elongation (Barry et al., 1993). Further, methylation of transposable elements can also silence read-through transcription from transposon promoters into neighbouring genes and thereby prevent inappropriate expression of those genes (Bender, 1998). Passage through tissue culture frequently results in reactivation of an inactive transposable element (Peschke and Phillips, 1991). This observation was first detected in the reactivation of transposable elements when Ac

activity was detected in maize plants regenerated from cultures derived from explants that contained no active Ac elements (Peschke et al., 1987). This study confirmed a link between activation of previously silent transposable elements following tissue culture and demethylation of the transposable element sequences.

III) Plant tissue culture and stress

Cultivation of plants *in vitro* is stressful and always carries the risk of genome variation, which might cause phenotypic variation. Recently, Cassells and Curry (2001) hypothesised that much of the variability expressed in tissue culture plants might be the consequence of, or related to, oxidative stress damage. This might be caused to the plant tissues during explant preparation and due to media and environmental factors in the propagation process. Typical stresses in plant tissue culture include high salt concentrations, water imbalance, mineral deficiency, excess in metal ions, overexposure to plant growth regulators, such as auxins and cytokinins, and the introduction of foreign genetic material during plant transformation (Figure 1.8) (Phillips et al. 1994; Skirvin et al., 1994; Cullis, 1999; Arnault and Dufournel, 1994).

A) Stress-induced variation of somaclonal cells

Genome variation in somatic tissue culture cells as a response to stress is a widespread phenomenon. This variation is also called somaclonal variation (Larkin and Scowcroft, 1981) and is not limited to any particular propagation technique or group of plants. In all organisms, spontaneous mutations occur from one generation to the next. However, somaclonal variation specifically describes the additional mutations in plants produced through stressful tissue culture (Bouman and de Klerk, 1997). Theoretically, all cells, organs or regenerated plants should be genetically identical in asexual plant tissue culture to the original explant from which plants have been generated. This unexpected source of variability was once hailed as a "novel source of

variation for crop improvement", but, due largely to its unpredictability as a breeding tool, enthusiasm for this application has diminished and somaclonal variation has lost much popularity in recent years (Karp, 1993).

A variety of morphological phenotypic variations, possibly due to somaclonal variation, occur in plants regenerated from tissue culture. It has been reported in ornamentals, plantation crops, vegetable and food crops, forest species and fruit trees (Rani and Raina, 2000). The economic consequences of somaclonal variation can be enormous especially in forest trees with long life cycles. Therefore, an analysis of micropropagated plants using a multidisciplinary approach, especially at genome level, is essential. Linacero et al. (2000) found hot spots of DNA instability in rye plants regenerated from immature embryos. At least 40% of the studied rye plants showed at least one variation and the number of mutations per plant was high ranging from 1 to 12. In a study using shoot-tip culture for banana micropropagation, six families of the cultivar 'Williams' showed no variation towards dwarfism but five families did produce dwarf variants (Israeli et al., 1996). Leroy et al. (2001) also found with callus of cauliflower using the microsatellite technology that only 6 out of 224 calli had stable original DNA patterns. Somaclonal variation was also found in *in vitro*-cultured beet plants (Sabir et al., 1992), red clover (Nelke et al., 1993) and *Brassica napus* (Poulsen et al., 1993). However, somoclonal variation has not always been confirmed in cultured plants. Using random amplified polymorphic DNA, Goto et al. (1998) found no genetically instability in micropropagated shoots of the Japanese black pine and interior spruce also showed no genetic instability in the embryogenic cultures when morphological characteristics and isozyme patterns were analyzed (Eastman et al., 1991).

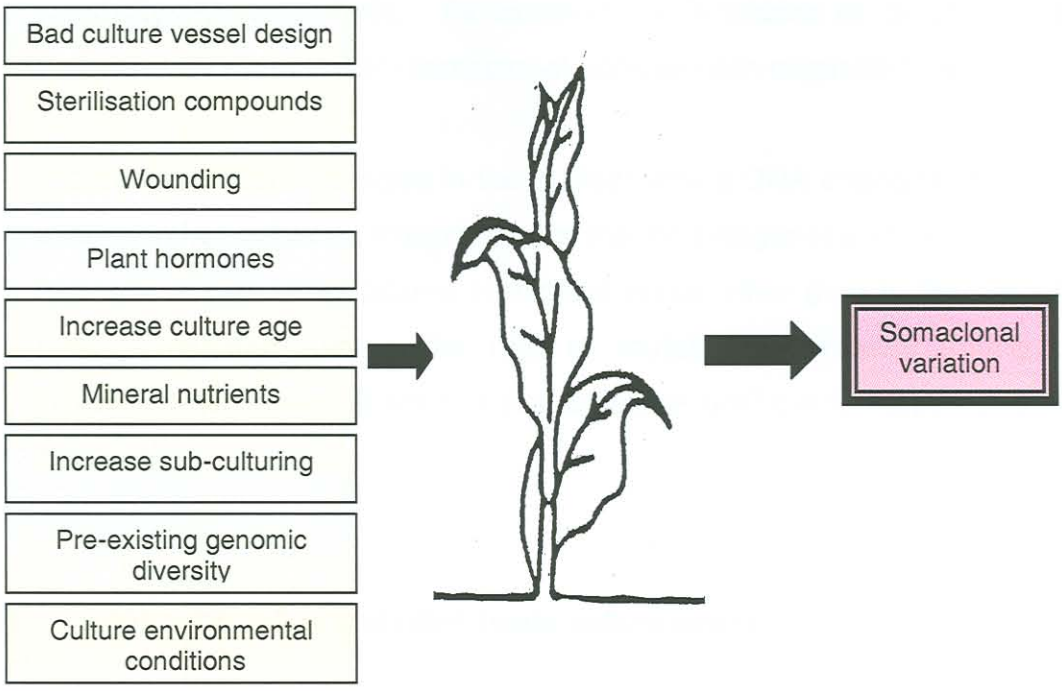

Figure 1.8: Stressful factors inducing genome modifications and plant phenotypes found as a consequence of plant tissue culture (McCoy et al., 1982; Brar and Jain, 1998; Ziv, 1991; Hagege, 1995).

Variation of somatic cells in plant tissue culture and its causes are still not well understood. Tissue culture plants are normally regenerated from a series of cell divisions in meristematic tissues and the apical meristem is formed during the early stages of plant embryo development from which the axillary meristem develops. An apical meristem may, however, originate also from non-zygotic cells, in particular from somatic or callus cells. Plants regenerated from adventitious meristems are often genetically different from the mother plant (Bouman and de Klerk, 1997) and somaclonal variation is therefore often associated with callus formation (Skirvin and Janick, 1976). The use of growth regulators and specifically the auxin-type compound 2,4-D, which induces callus formation, and also the cytokinin-type compound BA (6-benzylaminopurine) have been found to play an important role in the induction

of variability (Evans, 1988). As outlined by Smulders et al. (1994) and Bouman and de Klerk (1997) somaclonal variation can originate from:

- Programmed DNA changes in the explant where DNA changes are not an exception but rather an integral part of the morphogenesis of plants.
- Increase in rate of mutations in explant tissue other than in the apical or axillary meristem where the rate of mutations increases during cell divisions after the initial start of tissue culture until the formation of a new meristem.

B) DNA methylation and plant tissue culture stress

The stressful tissue culture environment also frequently alters DNA methylation patterns. Studies of regenerated plants with methylation-sensitive restriction enzymes have revealed both hyper- and hypo-methylation (Larkin and Scowcroft, 1981). Specifically, the hormone composition of the tissue culture medium can affect the level of DNA methylation in cultured cells. The antibiotics kanamycin and cefotaxime that are commonly used as selective agents in the production of genetically modified plants cause DNA hyper-methylation (Schmitt et al., 1997). Schmitt et al. (1997) also observed increased methylation in repetitive DNA, but the reversal of methylation was not obtained on removal of the antibiotics. Only plants grown from seeds in the progeny and in the absence of these antibiotics showed reduced methylation levels. Besides antibiotics, growth regulators added to the tissue culture medium also affect DNA methylation. Carrot cultures had reduced levels of methylation when grown on a cytokinin-containing medium (Arnholdt-Schmitt et al., 1991). Auxin-type plant growth regulators, such as 2,4-D and NAA, have also been implicated in alterations of DNA methylation. Methylation in carrot cell cultures increased during somatic embryogenesis when cultures were exposed to a high auxin concentration (Lo-Schiavo et al., 1989). However, alterations in methylation patterns in cultured cells have rather a tendency towards demethylation of cytosine residues (Jost and

Saluz, 1993). This might result from either imbalances between the activity of the enzymes involved in maintaining methylation, DNA replication and cell division or chromosome breakage and repair (Peschke et al., 1991).

C) Genetically modified plants and stress

Genome variations occur in genetically modified plants that have been derived from stressful plant tissue culture. In general, genetically modified plants are expected to integrate and express one or more foreign gene(s) in an otherwise unmodified genomic environment. Variation originating from the transformation process might be in addition to any changes arising from direct integration of the transgene. T-DNA integration after *Agrobacterium* infection can cause complex rearrangements and modifications around T-DNA/plant DNA junctions including base substitutions, duplication of border and genomic sequences and small nucleotide deletions (Ohba et al., 1995; Windels et al., 2001; Zheng et al., 2001; Stahl et al., 2002). In addition, increased chromosomal variation in transgenic barley plants, perhaps due to the additional stresses that occur during the transformation process, have also been reported (Choi et al., 2000a and b; Choi et al., 2000).

Known transformation procedures, such as *Agrobacterium tumefaciens* infection, which itself has been recently shown to be a stressful event (Ditt et al., 2001), particle bombardment and DNA transfer to protoplasts, include the use of cultured cells as an intermediate process in the regeneration of genetically modified plants. Labra et al. (2001) showed in rice the occurrence of genomic changes in genetically modified plants produced by infecting calli with *Agrobacterium tumefaciens*. Frequency of genomic variation from the original rice genotype was the highest in genetically modified rice recovered from protoplasts with the longest *in vitro* treatment, intermediate using *Agrobacterium* transformation and a callus phase, but the lowest with particle bombardment or intact cell electroporation.

Disagreement, however, exists about the most dominant reaction leading to variation. According to Sala et al. (2000), *in vitro* cell culture plays a more dominant role in inducing DNA changes than the insertion of the foreign gene itself. Bregitzer et al. (1998) also observed somaclonal variation in the progeny of genetically modified barley plants, but they found that the transformation procedure induced greater variation than the tissue culture process in the absence of transformation. However, regeneration of plants in the absence of a transformation process generally does not encounter the restraints imposed by the conditions used to select the genetically modified cells and also not the pre-existing genetic variability frequently present in the cultivar used.

D) Genetically modified plants and altered phenotypes

Phenotypic changes in genetically modified plants are well documented and include mainly alterations such as chlorophyll deficiencies, altered flowering time and reduced stem elongation. For example, phenotypic alterations found in transgenic rice that may not be due to the transgene integration or expression include longer flowering time, smaller plants and reduced fertility (Arencibia et al., 1998; Bao et al., 1996; Lynch et al., 1995). Altered growth characteristics were also found in genetically modified tobacco plants expressing the *gus* gene selfed over several generations (Caligari et al., 1993). The tobacco variety '*Samsun*' and genetically modified '*Samsun*' in which a rice cystatin gene (*OC-I*) had been introduced by *Agrobacterium tumefaciens*-mediated transform also showed a conditional phenotype. In the first year of this study, a conditional phenotype namely reduced elongation with significantly inhibited stem elongation, when grown under low light intensities were found. Physiological and biochemical analysis of these tobacco plants were done in a study carried out in collaboration with the research group of Prof. C. Foyer at Rothamsted Research (UK) (Van der Vyver et al., 2002, in press).

IV) Detection of stress-induced plant and genome variations

A range of different approaches is available for detecting genome variations and altered phenotypes. The available test methods differ, however, in their sensitivity, technical complexity, ease of use, and stage at which they can be applied. Screening at the morphological, cytological (chromosome number and structure), cytochemical (genome size), biochemical (proteins and isozymes), and molecular (nuclear and organelle genomes) levels provides a useful and often easy tool for detection of variation. Such detection of variation is specifically demanded in plant tissue culture, where severe environmental and chemical stresses might be imposed on plants (Rani and Raina, 2000; Cloutier and Landry, 1994).

A) Morphological and cytological screening techniques

Morphological screening and using chromosome structure for determination of genome and plant variation generally has the advantage of simplicity and the avoidance of any sophisticated analytical procedure. For chromosome structure, chromosome abnormalities can be screened before plant maturation in explants, such as roots, shoot apices, inflorescences, and also in protoplast and callus. Variation in chromosome structure can result in translocations, inversions, duplications and deletions of the plant genome (Karp, 1993). Tetraploid and hexaploid hybrids of potatoes have shown numerical and structural chromosome mutations (Waara et al., 1992).

In morphological screening, plant phenotypes are simply examined, often subjectively, via the description of easily detectable plant characteristics, such as plant form and structure. Change in appearance has been discovered in several tissue culture-derived graminaceous species, such as barley (*Hordeum vulgare*) and rye (*Secale cereale*) (Linacero and Vazquez, 1992), where 1% in barley and 50% in rye showed a morphological change, with chlorophyll deficiency as the most frequently observed change. Other morphological changes were the occurrence of an increased number of

flowers in each spikelet and production of poly-embryos. The frequency of morphological variation might differ, however, between plant cultivars and also appears to be dependent on the genotype.

B) Protein and DNA-based screening techniques

Highly discriminatory profiling methods using very sensitive molecular techniques ease the finding of minor genetic variations. Altered gene expression and changed genomic DNA patterns allow the evaluation of variation at the genome level. In contrast to morphological assessment, the assessment of variation at the genome level describes the internal make-up of a plant and identifies the variance in either the production of a plant protein expressed from certain regions of the DNA or in the DNA sequence. Figure 1.9 outlines the general process for plant identification by either protein or DNA analysis.

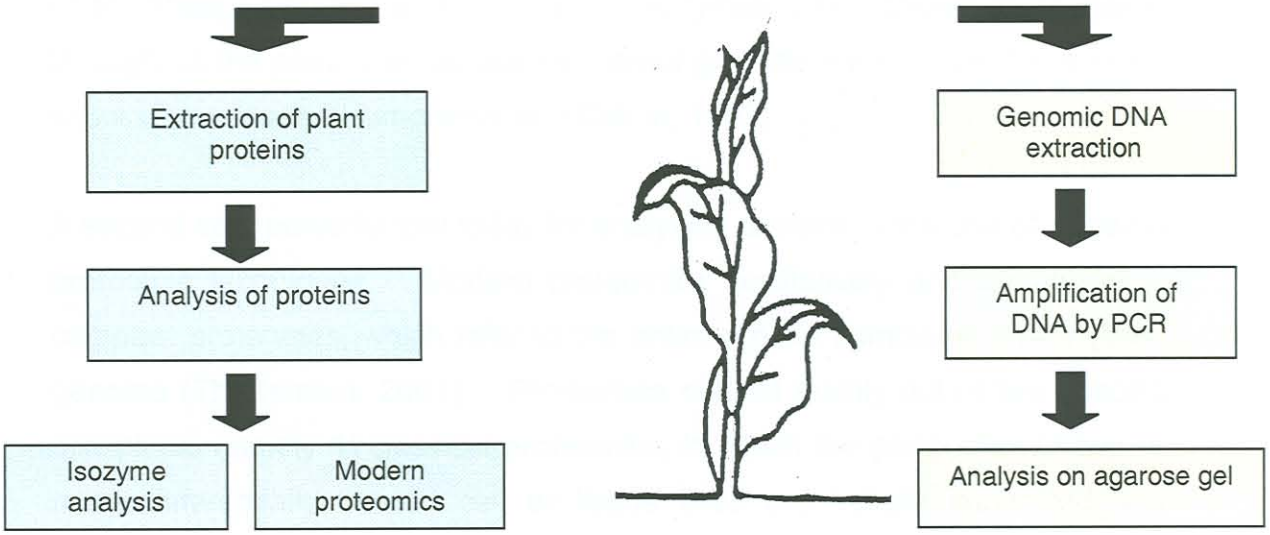

Figure 1.9: A general outline of the steps involved in plant identification by either protein or DNA analysis. As outlined below in detail, proteins are mainly analyzed as isozymes whereas DNA is analyzed by different amplification and subtraction techniques (O'Hanlon et al., 1999).

C) Protein-based screening techniques

Variation in protein patterns can be analysed as isozymes, which are different molecular forms of a protein actively controlling identical biochemical processes of a living cell. Technically, isozymes are separated in an electrical field supported in a polyacrylamide or starch matrix. A characteristic pattern of different isozyme bands called and "isozyme fingerprint" is visualised after staining. A difference between isozyme fingerprints derived from two types of plants is further called a "polymorphism". Enzymes most commonly measured include esterase (EST), glutamate oxaloacetate transaminase (GOT), endopeptidase (ENP), alcohol dehydrogenase (ADH), peroxidase (PER) polyphenol oxidase (POD) matrix (Bebeli et al., 1990). In the past, different isozyme patterns were found in different plant tissue, within and between plant species and between plant populations (Kawarasaki et al., 1996; Diaz et al., 1998; Elisiario et al., 1999). Variation in isozymes patterns were even found between individual *in vitro* grown shoots of Brassica species as well as in tomato, which has been exposed to abiotic stress (Samantaray et al., 1999; Lawrence et al., 1996). Isozymes, when shown to be stable throughout the plant, can be used to detect genome variation as it has been shown for grasses (Humphreys and Dalton, 1992).

A second very powerful tool today for analysing proteins is the use of modern proteomic techniques. Modern proteomics qualitatively and quantitatively compare proteomes, which refer to the entire protein complement of a given genome (Theillement, 2001). Proteomics consist mainly out of two specific disciplines namely 1) classical proteomics, in which the proteomes of two or more differentially treated cell or tissue lines are initially separated and visualized by 2D gel electrophoresis upon which proteins that differ in abundance between the gels are identified by mass spectrometry or 2) functional proteomics, where usually a subset of proteins has been isolated from a given starting material. Each protein in the subset has a common feature, which was used in the isolation procedure. Following some bioinformatics validation work, the common feature can give evidence of the function of each characterized protein (Klose, 1975; O'Farrell, 1975).

Drawbacks, however, for both classical and functional proteomics exist (Fey et al., 1997). Classical proteomics requires little or no prior knowledge about the proteome to be examined but cannot be use for example with all membrane proteins. Also, only proteins with sizes between 10 kDa and 100 kDa can migrate well in 2D gels, while proteins present in low copy numbers are not well detectable on 2D gels (Fey and Larsen, 2001). Functional proteomics in contrast require a good prior knowledge about the system to be studied. Regardless of any disadvantages presently existing in techniques available to study proteomes, it still remain a necessary approach to answer a great number of basic or applied questions. The recent success in discovering great amount of genes through modern genomics makes proteomics a necessary and complementary research field for deciphering the role and function of these newly revealed genes.

D) DNA-based screening techniques

In contrast to protein-based systems, DNA-based systems have the advantage that the DNA content of a cell is independent of environmental conditions, organ specificity or growth stage. Each cell of a living individual contains DNA as genetic material, and the DNA determines the individual characteristics via the control of protein synthesis in the cell. However, except for genome analysis in plant breeding and cultivar identification, DNA-based techniques to detect specifically stress-induced genome variations have been carried out in limited number to determine true-to-typeness of tissue culture-derived plants (Linacero et al., 2000; Skirvin et al., 1994).

Genome variations on the DNA level can be detected by techniques such as RFLP, AFLP, RAPDs and simple sequence repeats (SSRs), also known as microsatellites, (Figure 1.10) (Brown et al., 1990; Muller et al., 1990; Damasco et al., 1996; O'Hanlon et al., 2000). Among the recently introduced methods to detect genome variations, which still have to demonstrate their potential to reliably detect variations, are inter-simple sequence repeats (ISSR) (Albani and Wilkinson, 1998; Leroy et al., 2001) and representational difference

analysis (Powell et al., 1996; Cullis et al., 1999). A review article published by Cloutier and Landry (1994) outlines a variety of DNA-based techniques, which are applicable especially in plant tissue culture.

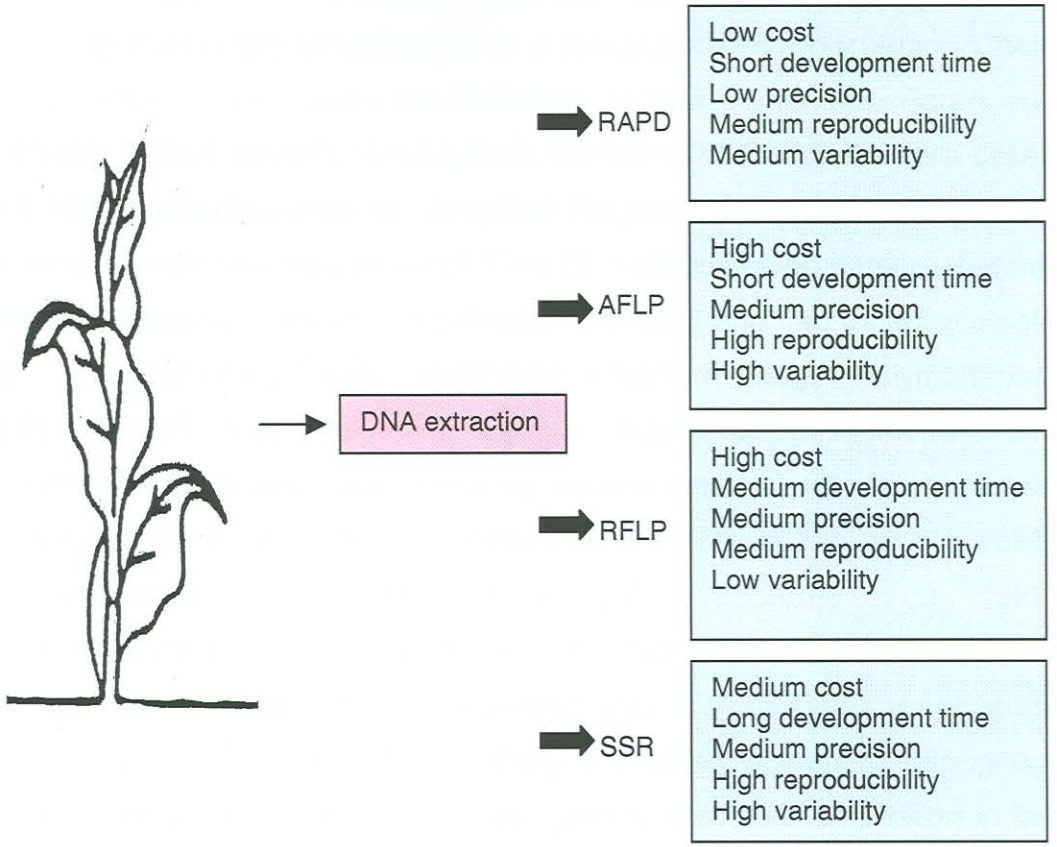

Figure 1.10: A summary of the qualitative characteristics of DNA based techniques to detect genome variations in plants. Cost = financial requirements to prepare a laboratory and obtain results; Development time = time required to develop genetic assays, depend on availability of primers; Precision = diversity present within a sample; Reproducibility = the ability to obtain the same genetic result for the same sample in repeated assays; Variability = inherent capacity of a DNA marker to reveal variation (adapted from O'Hanlon et al., 2000; Powell et al., 1996; Rafalski and Tingey, 1993).

D.1) RAPD, RFLP, AFLP and microsatellites

Characterisation of genome variation in plant tissue using Random Amplified Polymorphic DNA or RAPD analysis (Welsh and McClelland, 1990), which is

often referred to as DNA "fingerprinting", has been widely used to determine plant variation on the DNA sequence level (Heinze et al., 1996; Wolff, 1996). RAPD is possibly the simplest test of all recently applied DNA-based techniques consisting of the production of duplicate of segments of plant DNA and several million-fold amplification in a reaction called Polymerase Chain Reaction (PCR). To reduce the instability of RAPD markers, Paran and Michelmore (1993) recently developed a derivative of RAPD markers called SCAR (Sequence Characterized Amplified Region). SCAR are produced by PCR amplification driven by a pair of 20 to 25 nucleotide long primers that are derived by sequencing RAPD amplification products and can detect a single locus. In addition to RAPD, Restriction Fragment Length Polymorphism (RFLP), Amplification Fragment Length Polymorphism (AFLPs) and microsatellites have been also used by several research groups to detect DNA sequence variation in plant tissue culture (Mandolino et al., 1996; Chowdhury et al., 1994; Matthes et al., 2001; Vendrame et al., 1999; Ruskortekaas et al., 1994). Microsatellites, which are often moderately or highly repeated, consist of tandem repeated units of <6bp DNA repeats such as $(GA)_n$, $(GT)_n$, $(TG)_n$ or $(AAT)_n$. They are widely dispersed throughout eukaryotic genomes and are often highly polymorphic due to variation in the number of repeats. They are greatly informative and locus-specific in many plant species and they detect genome instability of a different type when compared for example with RAPDs. Microsatellites are isolated by screening genomic libraries, by genome walking with the labelled repeat of interest or from the analysis of expressed sequence tags (ESTs).

Technically, basic RFLP markers are detected by hybridisation of a probe, such as a cDNA clone, to restriction digested genomic DNA. In comparison, RAPDs use random ten base primers to generate large numbers of polymorphisms. AFLP markers are generated by PCR amplification of RFLPs. The AFLP procedure further uses adaptors being added to the ends of restriction fragments, and these adaptors are then used as primers in a PCR reaction. Amplified bands in AFLP are separated on a sequencing gel to identify differences in DNA band patterns. In addition, a second technique termed STS (Sequence Tagged Site) also derives from RFLP's. STSs are

short, unique sequences also amplified by PCR, but the primers are derived from sequence information of the ends of a genomic or cDNA clone that had produced an RFLP. An important advantage of STS is the elimination of the need for storage and distribution of clones (Cloutier and Landry, 1994). In order to accelerate gene tagging with molecular DNA markers a number of strategies have been in use for some time, which are all based on high resolution mapping of a target sites. These accelerated techniques all involve the pooling of DNA from individuals identical for a particular trait or phenotype and subsequently extracting the DNA as a pool to perform the comparative analysis (Churchill et al., 1993; Michelmore et al., 1991).

D.2) Representational difference analysis

Representational Difference Analysis (RDA) has attracted much attention especially in our group as a technique to identify genome variations in plants (Cullis and Kunert, 1999; Cullis and Kunert, 2000; Kunert et al., 2002; Vorster et al., 2002). RDA is a powerful DNA-based molecular subtractive technique to isolate labile hyper-variable DNA regions of the plant genome, which might have changed due to adaptation to the environment. Essentially, the method consists of a subtraction of all sequences that are held in common between two individuals, which might be morphologically identical, but differ for example significantly in their tolerance to environmental conditions or in the expression of certain morphological or biochemical characteristics. Technically, the RDA technology combines representation, subtractive hybridization, and kinetic enrichment. Representation means a production of the sub-population of DNA fragments derived from a given DNA population, such that the sequence complexity of the sub-population is lower than the sequence complexity of the initial DNA. Representations, which reduce complexity at least ten-fold over the complexity of the genome of higher organisms with genomes as complex as for grasses, are generally required for the success of the subsequent steps. Subtractive hybridization can be explained as eliminating similar sequences by hybridization between two representations and obtaining unique sequences present in only one of the

representations. Kinetic enrichment is based on the second order kinetics of DNA re-annealing. The rate of formation of double stranded DNA is higher for DNA species of higher concentration. The first round of RDA is mainly dependent on subtractive enrichment, but subsequent rounds do heavily rely on kinetic enrichment. In RDA, kinetic enrichment and subtractive enrichment are combined in a single step called hybridization/amplification.

The RDA technique has the potential, in comparison to the other DNA-based detection techniques, to give in a relatively short time period direct information about genomic losses, rearrangements and amplifications and also insertion of transposable elements into the genome (Lisitsyn et al., 1993). One of the most important advantages of RDA performed on genomes of different plants is its ability to scan in comparison to RFLPs, RAPDS and AFLPs up to 15% of the genome of most plants in each subtraction. The use of 300 random primers in a RAPD analysis for example would scan less than 1% of the same genome. Furthermore, RFLPs, RAPDS, AFLPs and microsatellites, generate random polymorphisms that are useful, for example, to define a population structure, but do not give an indication of the underlying causes of the population differentiation. The RDA technique is generally less suitable for such population studies, but is able to isolate and characterize any DNA sequences that might have changed in response to, or as an adaptation to, an environmental cue.

Another important advantage of RDA is its ability to preferentially isolate families of repetitive sequences that are unique to one of the compared genomes. Such families of repetitive DNA are homoplasy-free characters that can be converted into genetic markers for plant identification in a high throughput PCR-based assay (Nekrutenko et al., 2000). Identified labile regions in the plant genome by the RDA technique have also the potential to be applied as a genetic marker for a variable quantitative trait. A trait-linked genetic marker can be easily converted to a PCR-based marker applicable in studies to determine plant relationships based on traits or in a plant-breeding program.

E) Limitations of detection techniques

The applicability of each technique to detect stress-induced plant and genome variation is still limited. Morphological characteristics may vary widely with the environment and the growth stage of the plant. Some characteristics, however, which change through somaclonal variation, are obvious once plants mature. This includes a significantly changed plant structure or leaf discoloration and deformation. According to Karp (1993) several dis-advantages occur when using morphological screening or evaluation of chromosome structure in studying genome variation. Morphological variation may be epigenetic and not transmitted to the progeny and individual plants have to be grown to full maturity before any assessment can be completed. Cytology is further a time consuming staining technique and cannot be used to screen large numbers of plants or cultures. The absence of morphological variation or a normal chromosome complement does not mean that the plants are normal. Plants might carry small mutations, which are only manifested in the progeny of regenerated plants and single regenerated plants may also carry more than one small mutation, which may only segregate in the progeny.

Isozyme analysis, although easy to apply, has several general drawbacks (Karp, 1993). These include the dependence of isozyme expression on environmental conditions, the organ-specific presence of an isozyme and the often-limited amount of detectable polymorphism between individual plants. Further, isozyme analysis lacks a direct assessment of genomic variation at the genome level, which comprises the bulk of somaclonal variants in tissue culture plants.

RAPDs are normally found to be easy to perform but have the major disadvantage that reproducibility is difficult to achieve between different laboratories and often even between different people in the same laboratory (Jones et al., 1997). Constant detection of identical DNA amplification products has to be confirmed by several-fold repeated experiments preferably by different people. There are also several reports on the importance of *Taq*

polymerase and the thermocycler used in the PCR reaction for RAPDs and variability in RAPD profiles due to the use of different brands of both (Khandka et al., 1997; This et al., 1997). For example, different brands of DNA *Taq* polymerase amplify differently, which results in varying profiles of DNA amplification products. Skroch and Nienhaus (1995) examined the impact of this irreproducibility on the scoring of RAPDs. When expressed as the percentage of RAPD bands scored that were also scored in replicate data, only 75% reproducibility was obtained for 50 RAPD primers. Both RAPDs and AFLPs simply compare the DNA from any number of different samples and can be used to detect the level of difference between them. In both cases only those differences specific to a particular primer set are detected in any reaction. Thus, if the material is only different at a few sites within the genome (closely related) then a large number of primers have to be used in order to detect variation. For example, in experiments with flax, the use of 300 different RAPD primers only covered about 1% of the genome (Oh and Cullis; personal communication).

Microsatellites have clearly demonstrated their merits in population studies determining the gene flow between plant populations but have limitations to identify any kind of unknown variable region in the plant genome. They suffer from a similar drawback as RFLPs, namely, they need to be isolated and then to be characterized. RFLP also requires both large amounts of DNA and the isolation of informative probes that yield differences between the sources of the DNA. Identified genome changes detected with these two techniques are also rarely directly linked to a trait. Both ISSR and RDA, although known for some time, have not been widely evaluated as a technique. For RDA this is partially due to its complexity and therefore one the objectives of the following study was to evaluate the potential of the RDA technique for identification and characterization of genome differences possibly induced in genetically modified plants.

Conclusions

It is been well established that an organism needs to adapt to a stressful environment in order to survive. The majority of these adaptive responses for survival are at the gene expression level aimed to overcome a short period of stress. They are not designed to generate a permanent genetic change in the DNA sequence of the plant genome. A relative small number of reports indicate, however, that such DNA sequence changes can occur in response to exposure of a plant to a stressful environment. Therefore, there is a need to study such possible stress-induced DNA sequence changes in greater detail to which this study has contributed by comparing the genomes of a unstressed plant and a plant following exposure to a stressful event such as plant transformation, which includes a tissue culture process. Several molecular techniques have been further applied to detect such changes in the genome. But, so far these techniques have yielded little or no specific sequence information of the genome parts that might change under stress. Consequently, there is also a need to evaluate new techniques for identification, isolation and characterization of such susceptible genome regions that vary following stress exposure. The results reported here have further contributed to this end by evaluating the RDA technique. Finally, there is a need to develop markers for detection of these possible sequence changes, which might be ultimately related to either beneficial or detrimental phenotypical plant alterations under stress. This study therefore focuses on experiments to possibly relate any detected genome variation to a stressful condition.

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